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13. ABSTRACT *(Maximum 200 words)*

The purpose of this grant is to examine the role of the cyclin D1 gene, and related genes, in multistage breast carcinogenesis. These studies may provide new biomarkers and suggest novel strategies for the prevention and treatment of breast cancer.

During the past year two studies were completed. The first, which is in press in Carcinogenesis indicates that carcinogen-induced rat breast tumors display dysregulation in the expression of cyclins D1 and E. Since similar changes are seen in human breast cancers, the rat model may be useful for studying the role of dysregulated expression of these cyclin genes in breast cancer progression and prevention. The second study, which was recently published in Oncogene, indicates that overexpression of cyclin D1 in human mammary cells can inhibit rather than stimulate cell growth. The underlying mechanisms and the implications of these findings are the subjects of current studies.

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I. Bernd Weinstein 8/6/95

PI - Signature Date

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(5) INTRODUCTION

Nature of the problem. Although there is considerable evidence that the majority of human breast cancers are caused by environmental factors (including dietary factors) and reproductive factors, rather than hereditary factors, the specific causes of human breast cancer are not known with certainty. Furthermore, although exciting progress has been made in identifying mutations and or aberrant expression of cellular oncogenes and tumor suppressor genes in human breast cancers, the precise mechanisms responsible for the uncontrolled proliferation of breast cancer cells, the apparent genomic instability of malignant breast tumors, and the often relentless course of tumor progression, are poorly understood at the present time.

Background. Cyclins are a recently identified family of proteins that regulate the passage through the G1, S, G2 and M phases of the cell cycle (for review see 1-3). These proteins complex with specific cyclin-dependent serine-and threonine-protein kinases (CDKs), thereby regulating the activity of these kinases. This process is further modulated by the phosphorylation and dephosphorylation of CDK proteins by specific protein kinases and phosphatases, and by specific inhibitory proteins called CDKIs. At least 8 distinct cyclin genes have been identified in the human genome and at least five CDKs (CDK 1-5) form complexes with these cyclins. Based on their conserved sequence motifs with cyclins in other species and their patterns of expression and apparent functional roles during the cell cycle, they are grouped into three categories: G1 cyclins, including cyclins D1-3, C and E; the S-phase cyclin, cyclin A; and G2/M phase cyclins, cyclins B1 and B2. Cyclin B is the best characterized mammalian cyclin. It complexes with CDK1 to regulate both mitotic entry and exit. It is not known whether cyclin B1 and B2 are interchangeable. The precise functions of the other cyclins in the cell cycle is not as well defined in mammalian cells as in lower organisms. After stimulating quiescent cells by growth factors, cyclin D1 is expressed maximally during mid to late G1, although it appears to be expressed at a constant level in continuously dividing cells. Cyclin D1, and cyclins D2 and D3, associate with CDK kinases thereby activating their activities. The Rb protein is a target for phosphorylation by these complexes in vitro (12). There is conflicting evidence, however, on the ability of cyclin D1 to complex directly with the Rb protein. It is not known whether all of the biologic effects of cyclin D1 are mediated via the Rb protein.

Several lines of evidence implicate the role of cyclins D1 and E in human breast cancer (see 1-4). 1) The cyclin D1 gene (originally termed Prad1), is located at chromosome 11q13. This locus is frequently amplified in human breast cancers. In recent studies, described in greater detail below, we have demonstrated amplification and increased expression of cyclin D1 in a subset of primary human and breast cancer cell lines. 2) The cyclin E gene was found to be amplified and expressed at a high level in a human breast cancer cell line (1-4). 3) Increased expression, and abnormalities during the cell cycle, in the expression of cyclins D1 and E have been described in human breast tumors and cell lines (1-4). It is of interest that the increased expression of cyclin D1 did not always correlate with amplification (7,8).

Our laboratory has previously described amplification and increased expression of cyclin D1 in human tumors of the liver and esophagus (3-6). We have also demonstrated that stable overexpression of cyclin D1 shortens the G1 phase of the cell cycle and enhances malignant cell

transformation (4). These studies from our laboratory, coupled with the other evidence (cited above) of abnormalities in cyclins in human breast cancers, provide the basis for this grant.

Purpose of the present work. The overall purpose of this work is to examine the hypothesis that amplification and/or increased expression of the cyclin D1 gene plays an important role in multistage breast carcinogenesis by enhancing the process of tumor progression. These studies might also provide new biomarkers and diagnostic tools to more precisely detect and stage breast tumors. This approach could also lead to the development of agents that inhibit the action of specific cyclins or cyclin-dependent protein kinases in human tumors, and thus lead to new strategies for breast cancer chemoprevention and therapy. If abnormalities in the expression of cyclin genes enhance genomic instability, then such inhibitors might specifically block the process of tumor progression and the emergence of hormone independent and drug resistant variants of breast tumors.

Methods of approach. As discussed above, the cyclin D1 gene is frequently amplified and overexpressed in primary human breast cancers and breast cancer cell lines, but the functional and prognostic significance of this finding is not known. We are using well defined normal human mammary epithelial and human breast cancer cell culture systems to analyze the role of cyclin D1 in cell cycle control, gene expression and amplification, cell transformation and tumorigenicity. Similar studies are also being done with cyclin E. A major strategy employed during the past year was to utilize gene transfer methods to develop derivatives of normal mammary epithelial cells that stably overexpress either cyclin D1 or cyclin E and then examine possible effects on growth control, differentiation and various cell cycle parameters (6).

(6) BODY

(a) Deregulated expression of cyclin D1 and other cell cycle-related genes in carcinogen-induced rat mammary tumors.

As discussed in the above **Introduction** there is accumulating evidence that human breast cancers display abnormalities in the expression of genes that regulate the cell cycle, especially cyclins D and E. Carcinogen-induced breast tumorigenesis in rats has provided a valuable model for understanding the process of mammary carcinogenesis. However, possible changes in cyclin gene expression had not been studied in this model system. Therefore, during the past year we analyzed the expression of cyclins D1, E and A and other cell cycle-related proteins in a series of nine *N*-methyl-*N*-nitrosourea-induced primary rat mammary tumors (Figure 1). Western blot analysis revealed a 10- to 15-fold increase in the level of cyclin D1 protein in most (7/9) of the tumors, when compared with normal rat mammary gland. The two tumors that did not show this increase also displayed negligible levels of the retinoblastoma protein. A moderate increase, 1.5- to 2-fold, in the level of cyclin E was observed in four tumors and three tumors displayed abnormal low molecular weight cyclin E-related proteins. None of the tumors showed amplification of the cyclin D1 or E genes when studied by Southern blot analysis. All nine tumors showed a 2- to 6-fold increase in the level of cyclin A protein. Most of the tumors also displayed a marked increase in levels of the CDK2 and CDK4 proteins. These changes did not appear to be simply a consequence of increased cell proliferation as assessed by proliferating cell nuclear

antigen analysis.

Thus, aberrant expression of cyclins and other cyclin-related genes occurs frequently in mammary tumorigenesis in both rodents and humans. Since this rat model system resembles human mammary carcinogenesis, with respect to abnormalities in cyclin gene expression and in other respects, it may be useful for mechanistic studies on deregulated expression of cyclin genes. It may also be useful for screening new drugs that might block mammary carcinogenesis by inhibiting the function of specific cyclins. A paper describing these findings is now in press in the journal Carcinogenesis (5).

(b) Stable overexpression of cyclin D1 in a human mammary epithelial cell line prolongs the S-phase and inhibits growth.

Several laboratories have studied amplification and expression of cyclin D1 in mammary tumors and cell lines. However, only a few studies had investigated cyclin D1 expression at the protein level (7,8) and there had been no previous studies on the growth characteristics of mammary epithelial cells engineered to stably overexpress an exogenous cyclin D1 sequence. Therefore, in our initial studies we examined in parallel cyclin D1 amplification and expression, at both the mRNA and protein levels, in two human epithelial cell cultures originally established from normal human mammary epithelium and in five cell lines originally established from human breast carcinomas.

Southern blot analysis (Figure 2A and B) indicated that cyclin D1 was highly amplified (about 10-fold) in the MDA-MB-134 and MDA-MB-330 carcinoma cell lines, when compared to the normal mammary epithelial cell line Hs578Bst. A moderate level of cyclin D1 amplification (about 2- to 3-fold) was seen in the MCF-7, ZR-75-1 and T-47D breast carcinoma cell lines. The HBL-100 cell line, which was originally established from apparently normal human mammary epithelium but expresses the SV40 large T antigen and is partially transformed, did not show amplification of cyclin D1.

Northern blot analyses (Figure 3A) indicated a very high level of cyclin D1 mRNA in the MDA-MB-134 cells, a moderately high level in the Hs578Bst, MCF-7, and ZR-75-1 cells, a somewhat lower level in the T-47D cells and an extremely low level in the HBL-100 cells. The MDA-MB-330 cell line was not examined in this study. The ethidium bromide staining which was done to control for RNA loading (Figure 3B) suggested that the relative level of cyclin D1 mRNA in MDA-MB-134 cells is even higher than that revealed in Figure 3A. Western blot analysis (Figure 2C) indicated a very high level of cyclin D1 protein in the MDA-MB-134 cells, which also displayed the highest level of cyclin D1 amplification and mRNA expression (Figures 2A and 3A). A moderate level of cyclin D1 protein was present in the MDA-MB-330 cells, a low level in the ZR-75-1 and MCF-7 cells, and trace or undetectable level in the T-47D carcinoma cell line. The Hs578Bst and HBL-100 cells originally established from normal mammary epithelial cells also displayed only trace amounts of cyclin D1 protein (Figure 2C). Thus in this series of cell lines there was a general, but not uniform, correlation between cyclin D1 gene amplification and the level of expression of cyclin D1 mRNA and protein.

The above study indicated that the cyclin D1 gene was neither amplified nor expressed at significant levels, at both the mRNA and protein levels, in the HBL-100 cell line. Thus, we decided to stably overexpress an exogenous cyclin D1 cDNA in this cell line by using a retrovirus-derived vector, to determine possible effects on growth, cell cycle parameters and gene expression. In previous studies we used the same retrovirus derived vector (pMV7-CCND1) to stably overexpress cyclin D1 in rat R6 fibroblasts (4). Following transduction and selection for neomycin resistance a number of vector control and cyclin D1 clones were obtained. As shown in Figure 3C, cyclin D1 protein was not detected by western blot analysis of proteins extracted from seven neomycin resistant vector control clones. However, appreciable but varying amounts of cyclin D1 protein were detected in nine neomycin resistant clones derived from HBL-100 cells transduced with the PMV7-CCND1 vector (Figure 3C). We chose three vector control clones (HBL-vt no. 1, HBL-vt no. 2, HBL-vt no. 3) and three cyclin D1 clones (HBL-cycD1 no. 2, HBL-cycD1 no. 5, HBL-cycD1 no. 16) for further studies. Densitometric analysis of western blots indicated that the HBL-cycD1 no. 2 clone expressed about a 7-fold increase in cyclin D1 protein, and the HBL-cycD1 no. 5 and no. 16 clones expressed about a 5-fold increase, when compared to the vector control clones. Immunofluorescence analysis of intact cells using a cyclin D1 specific antibody confirmed the high level of expression, and also demonstrated nuclear localization of the cyclin D1 protein in the latter three clones (data not shown). Northern blot analysis demonstrated a high level of cyclin D1 mRNA, of the expected size (4.5kb), in these three clones, especially clone no. 2, but cyclin D1 mRNA was not detected in the three vector control clones (Figure 3A).

To examine the growth properties of the cyclin D1 overexpressor clones, several parameters were studied including exponential doubling time, saturation density, anchorage-independent growth, and tumorigenicity in nude mice (see Table 1). When grown in 10% FCS the parental HBL-100 cells and the three vector clones showed similar doubling times (about 28-29h). The doubling times of the three cyclin D1 overexpressor clones were increased to about 34-36h. It is of interest that similar values were obtained when the vector control or overexpressor clones were grown in the presence of 1% FCS (Table 1). Thus HBL-100 cells have a relatively low requirement for serum and this is also true of the cyclin D1 overexpressor clones. The saturation density of the three cyclin D1 clones was also reduced when compared to the vector control clones. The decrease was greatest with overexpressor clone no. 2 which had the highest expression of cyclin D1 (Table 1 and Figure 3C). The cloning efficiencies of the three overexpressor clones in monolayer culture were also markedly reduced when compared to the HBL-100 parental and three vector control cell lines (Table 1).

The HBL-100 parental cells and the vector control derivatives were able to grow in soft-agar. The three overexpressor clones showed a decreased cloning efficiency in agar when compared to the control cells (Table 1). In addition, the colonies formed by the cyclin D1 overexpressor clones were flatter and more monolayer-like than those formed by the vector control cells (data not shown). When these cell lines were tested for tumor formation in nude mice, none of the cyclin D1 overexpressor clones formed tumors, whereas the parental HBL-100 cell line and one of the three vector control clones (HBL-vt no. 3) did form tumors (Table 1).

Table 1 Growth properties of cyclin D1 overexpressing HBL-100 cells*

	Growth in monolayer culture					Growth in agar	Tumorigenicity in nude mice
	Doubling time(h)		Saturation density($x10^6$)		Cloning		
Cell line	1%FCS	10%FCS	1%FCS	10%FCS	Efficiency(%)	Efficiency(%)	
HBL-100	29.8	28.8	3.2	3.1	13.0	9.6	2/2
HBL-vt#1	28.8	28.8	3.0	3.0	17.6	8.4	0/2
HBL-vt#2	28.5	27.6	3.2	3.0	18.0	7.2	0/2
HBL-vt#3	29.4	28.8	3.2	3.4	26.0	6.5	2/2
HBL-cycD1#2	36.8	36.0	0.5	0.9	7.1	5.0	0/2
HBL-cycD1#5	34.1	33.6	1.5	2.0	5.2	4.1	0/2
HBL-cycD1#16	34.9	33.6	1.5	2.3	9.2	5.3	0/2

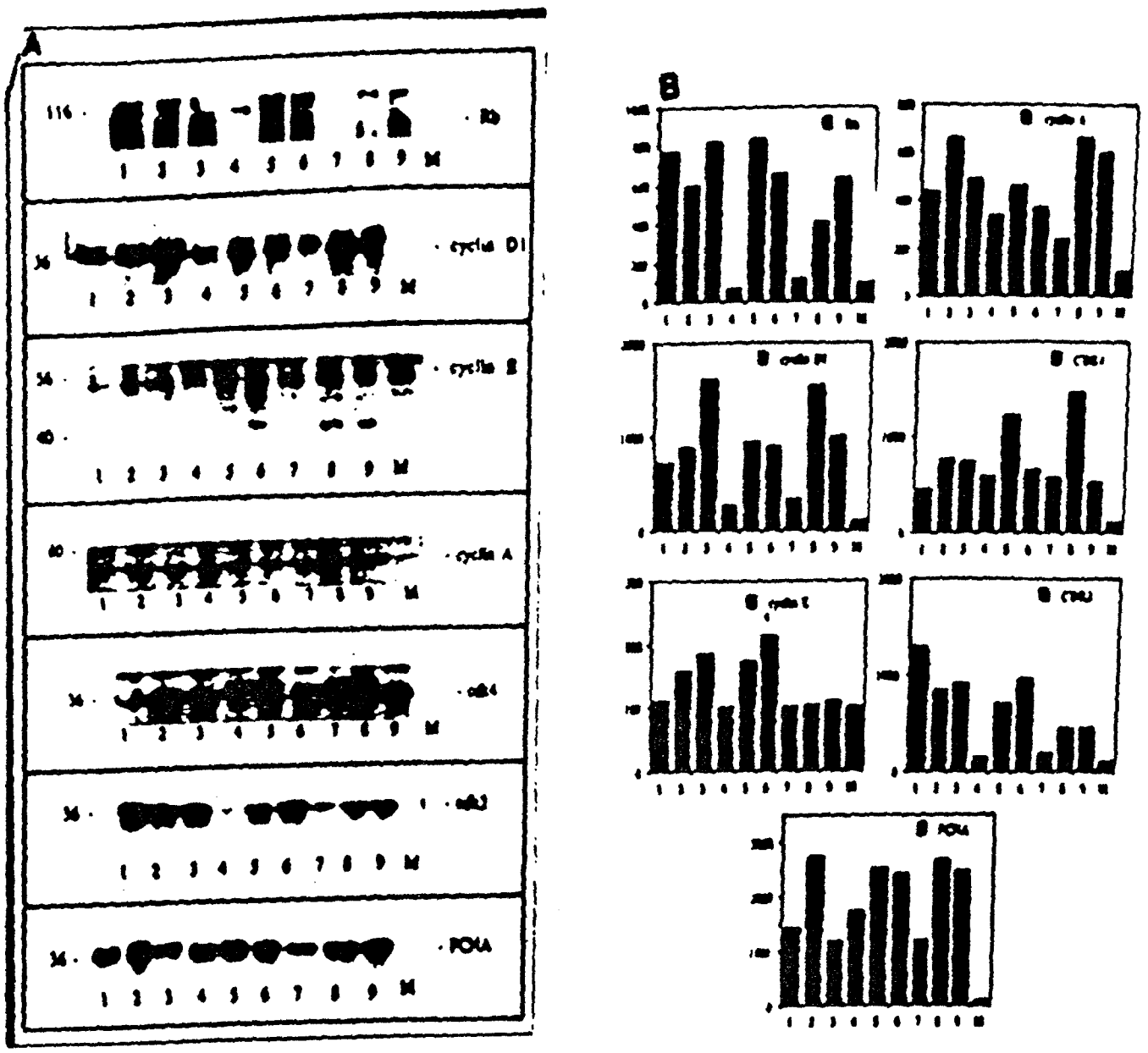

Figure 1. Deregulated expression of cyclins and cyclin-related proteins in rat mammary tumors. (A) Protein extracts from NMU-induced rat mammary tumors (1-9) or from normal rat mammary gland (M) were used to perform Western blot analysis as described in the text, using antibodies to the indicated proteins. The position of molecular weight markers in kilodaltons (kDa) is indicated on the left. Faint bands for cyclin D1, PCNA, Rb protein and the two CDKs were seen on the original Western blots of the normal mammary gland sample (B). The relative intensities of the corresponding bands were determined by densitometer analysis.

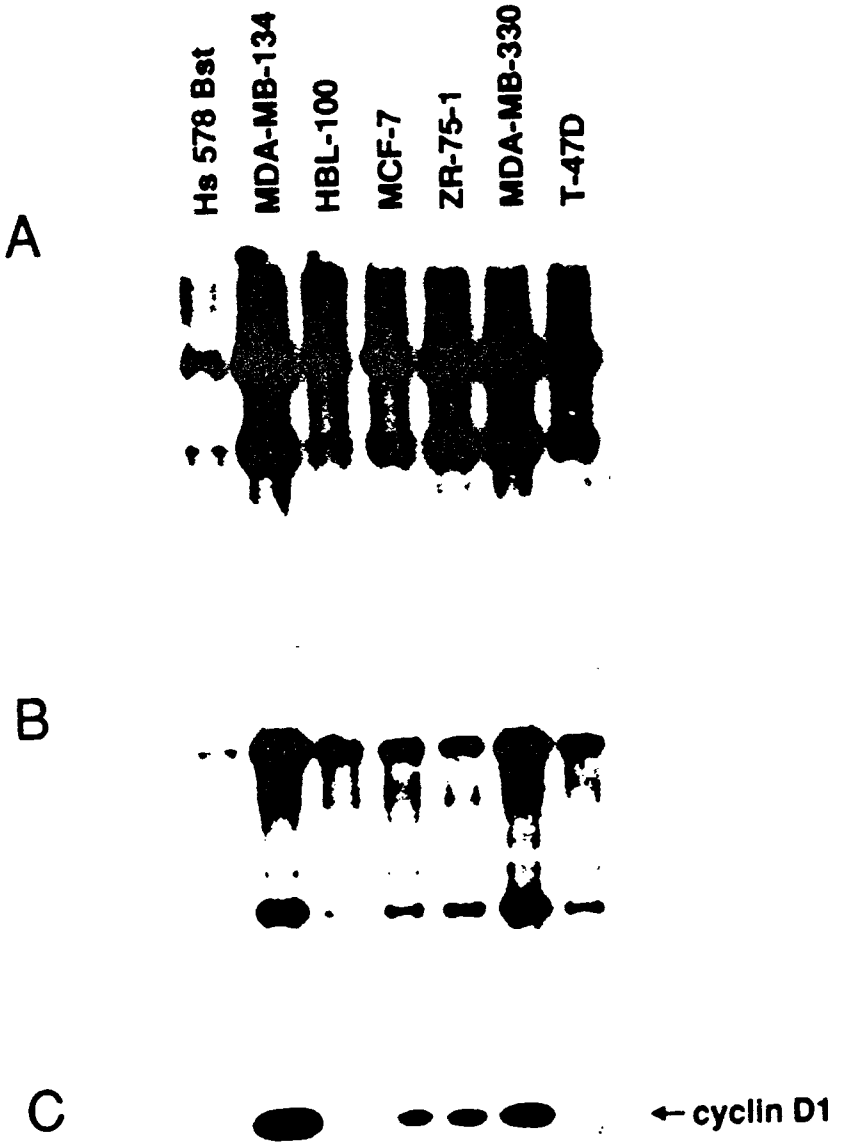

Figure 2. (A) and (B) Southern blot analysis of cyclin D1 in human mammary epithelial and breast carcinoma cell lines. Genomic DNA (5 μg each) was isolated from each of the indicated cell lines and digested with either EcoRI (A) or HindIII (B). Digested samples were electrophoresed, transferred to a membrane and hybridized with a ^{32}P-labelled cyclin D1 probe. (C) Western blot analysis of protein extracts from the same cell lines using a cyclin D1 antibody.

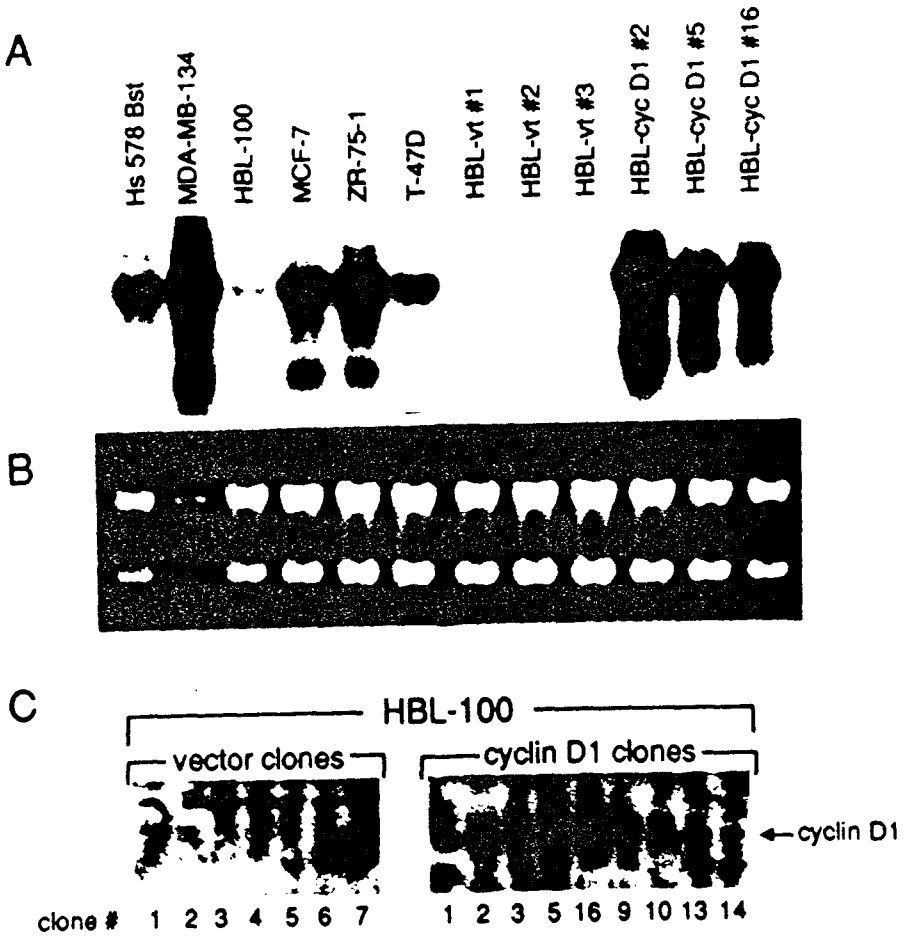

Figure 3. (A) Northern blot analysis of cyclin D1 mRNA. Total RNAs were isolated from the indicated cell lines and derivatives of the HBL-100 cell line. Samples (10 μg each) were electrophoresed, transferred to a membrane and hybridized with a ^{32} P-labelled human cyclin D1 cDNA probe. Three vector control clones (HBL-vt no. 1, no. 2 and no. 3) and three cyclin D1 overexpressor clones (HBL-cyc D1 no. 2, no. 5 and no. 16) are also shown. (B) Ethidium bromide staining of the membrane is shown as a control for RNA loading. (C) Western blot analysis showing the levels of expression of cyclin D1 protein in a series of HBL-100 vector control and cyclin D1 overexpressing clones.

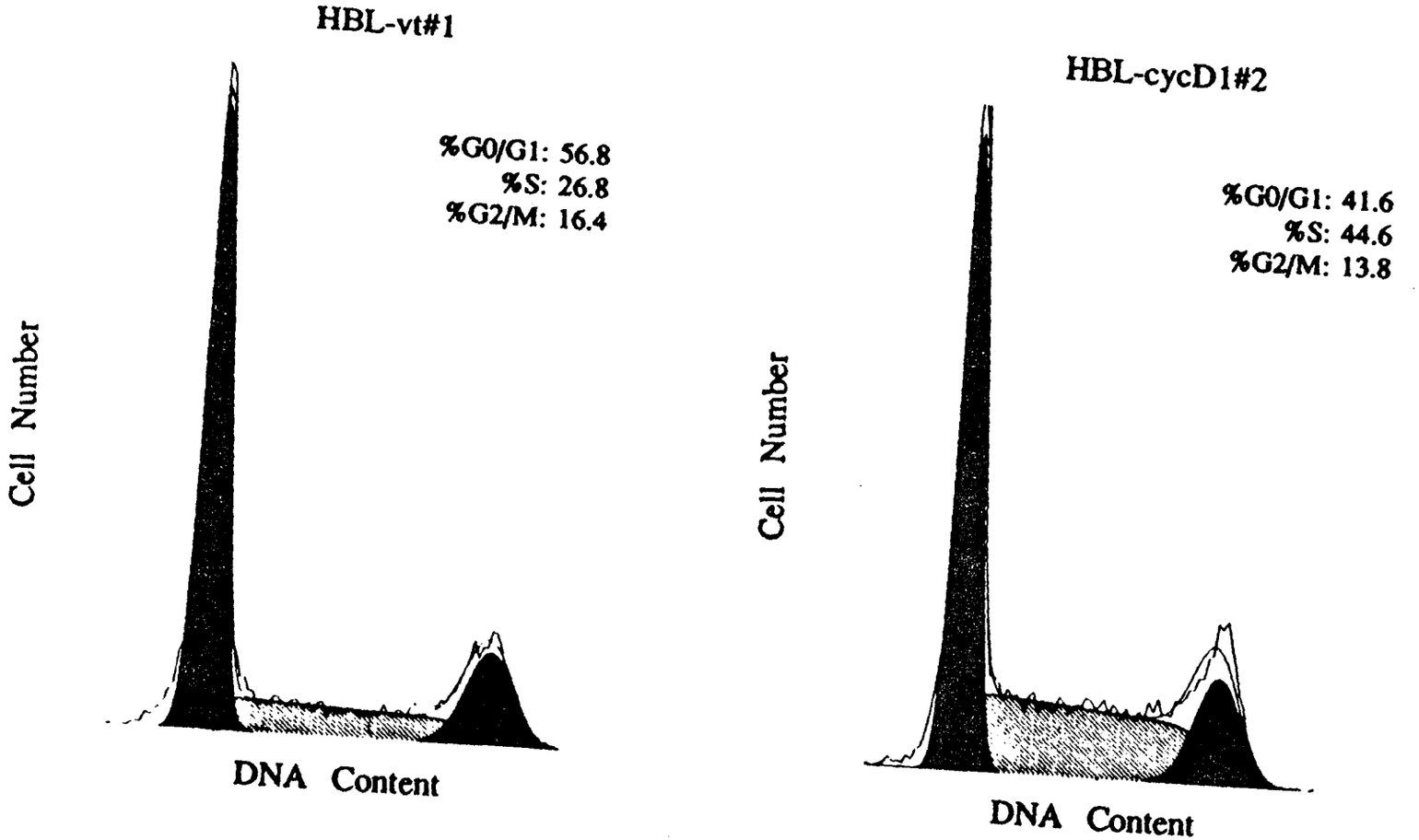

Figure 4. Flow cytometry profiles of HBL-vt no. 1 and HBL-cycD1 no. 2 clones.

The above studies clearly indicate that an approximately 5-7 fold increased expression of the cyclin D1 protein in HBL-100 cells markedly inhibited the growth of these cells, by several parameters, and also decreased the tumorigenicity of these cells. A possible explanation is that overexpression of cyclin D1 in this cell line increases the production of an extracellular growth inhibitory substance. In preliminary experiments, however, we have been unable to demonstrate that conditioned medium obtained from the HBL-100 cyclin D1 overexpressor clones inhibits the growth of the parental HBL-100 cells (unpublished studies).

It was of interest to examine HBL-100 control and cyclin D1 overexpessor cells obtained from exponentially dividing non-synchronized cultures by flow cytometry after "tagging" the DNA of these cell with propidium iodide. Representative flow cytometry profiles for the vector control cell line HBL-vt no. 1 and for the cyclin D1 overexpressor cell line HBL-cycD1 no. 2 are shown in Figure 4. Overexpression of cyclin D1 decreased the percent of the total cell population that is in the G0/G1 phase, increased the percent that is in S-phase, and had no consistent effect on the percent in the G2/M phase. This general pattern was also seen in additional experiments (data not shown). Thus, the increased doubling time of the cyclin D1 overexpressor clones appears to be due, mainly, to a lengthening of the duration of the S-phase. It is of interest that the MDA-MB-134 cell line which displays amplification and high expression of the endogenous cyclin D1 gene (Figure 2) also grows slowly.

Thus, derivatives of the HBL-100 human mammary epithelial cell line that stably express high levels of cyclin D1 display an increased doubling time, decreased saturation density, decreased cloning efficiency, decreased anchorage-independent growth, an increased fraction of cells in the S-phase, and decreased tumorigenicity. Thus, increased expression of cyclin D1 in this cell line markedly inhibits rather than enhances growth, which may be due to the prolongation of S-phase. These findings were recently published in the journal Oncogene (6).

(7) CONCLUSIONS

During the first year of this research project we have completed two specific studies that are directly related to the original goals of this research proposal.

The first study, which is now in press in the journal Carcinogenesis, demonstrates for the first time that carcinogen-induced rat mammary tumors display abnormalities in the expression of several cell cycle-related genes, especially cyclins D1 and E. Since similar changes are seen in human breast carcinomas the rat system may be a useful model for studying the role of these abnormalities in breast cancer causation, prevention and treatment.

The most important finding in the second study, which was recently published in the journal Oncogene (6), is that stable overexpression of an exogenous cyclin D1 cDNA in the HBL-100 human mammary epithelial cell line markedly inhibits rather than enhances the growth of these cells. This effect is not confined to the HBL-100 cells since we have found that overexpression of cyclin D1 also inhibits the growth of another human mammary epithelial cell line Hs578Bst and the mouse mammary epithelial cell line HC11 (studies in progress). Nevertheless, further studies are required to determine to what extent our findings can be generalized to other mammary

epithelial and breast carcinoma cell lines.

These findings are in contrast to our previous results in which stable overexpression of cyclin D1 in R6 rat fibroblasts enhanced growth and tumorigenicity (4). Thus, the effects of increased expression of cyclin D1 on cell growth are a function of the context of particular cell types, presumably reflecting the levels of expression of other cellular genes. Since the derivatives of HBL-100 cells that overexpress cyclin D1 have a prolonged S-phase it is possible that in some cell types increased levels of cyclin D1 inhibit critical events related to DNA synthesis. Therefore, in current studies we are examining the levels of expression of PCNA and other proteins involved in DNA synthesis, the Rb tumor suppressor gene, various other cyclins, and a series of cyclin dependent kinase inhibitory proteins (CDKIs), to determine their roles in modulating the effects of cyclin D overexpression in mammary epithelial and breast carcinoma cell lines and tumors.

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